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(54) Title: METHODS OF DETERMINING AN INCREASED RISK OF A WOMAN CARRYING A DOWNS SYNDROME		
(57) Abstract		
There are provided methods for assaying biological specimens for one or more of leptin, prorenin or renin in order to provide predictive information about the likelihood of a woman carrying a Downs Syndrome affected fetus.		

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METHODS OF DETERMINING AN INCREASED RISK OF A WOMAN CARRYING A DOWNS SYNDROME

5 **Field of the Invention**

 The present invention relates to assay methods which allow for the detection, and quantitation, of analytes; such as, leptin and/or prorenin and/or renin, in biological samples, such as blood or urine from a pregnant woman, which are associated with an increased risk that the pregnant woman's fetus has Downs Syndrome.

10 **Background of the Invention**

 Trisomy 21, commonly known as Downs syndrome, is characterized by an extra copy of chromosome 21. People afflicted with Downs syndrome have severe mental retardation, reduced life expectancies, and abnormal immune responses that predispose them to serious infections as well as thyroid autoimmunity. Further, 40% of Downs syndrome patients have congenital heart disease and a 10 to 20-fold increased risk of developing leukemia relative to the general population. All Downs syndrome patients older than 40 develop neuropathological changes characteristic of Alzheimer's disease.

 Prenatal tests to detect aneuploidy, such as trisomy 21, by amniocentesis or chorionic villus sampling (CVS) have been available since the late 1960s. Amniocentesis is the most common invasive prenatal diagnostic procedure. In amniocentesis, amniotic fluid is sampled by inserting a hollow needle through the mother's anterior abdominal and uterine walls into the amniotic cavity by piercing the chorion and amnion. It is usually performed in the second trimester of pregnancy. CVS is performed primarily during the first trimester, and involves collecting cells from the chorion which develops into the placenta.

30 Another invasive prenatal diagnostic technique is cordocentesis or percutaneous umbilical cord blood sampling, commonly known as fetal blood sampling. Fetal blood sampling involves obtaining fetal blood cells from vessels of the umbilical cord, and is performed about the 20th gestational week.

Amniocentesis is used selectively because it presents a risk of about 1% of inducing spontaneous abortion. CVS and fetal blood sampling carry a similar or higher risk of inducing abortion, and there is also concern that these procedures may lead to fetal limb malformations in some cases. Thus, amniocentesis, CVS and fetal blood sampling are procedures that are only employed if a pregnancy is considered at high risk for a serious congenital anomaly. Thus, some means is required to select those pregnancies that are at a significant risk of Downs syndrome to justify the risks associated with invasive prenatal diagnostic procedures, such as amniocentesis, CVS and fetal blood sampling.

Prior to 1983, the principal method for selecting pregnancies that had an increased risk for Downs syndrome was based on maternal age, that is, the older the age of the mother, the higher the risk that the fetus would be affected by Downs syndrome. In 1974, biochemical screening for neural tube defects by measuring alpha-fetoprotein (AFP) in serum began. In 1984, the use of the AFP screen was additionally adopted for the detection of Downs syndrome. Since the early 1990s, a multiple marker blood test has been used to screen for this disorder. A common version of this test is the three marker triple test. The triple screen measures AFP, human chorionic gonadotropin (hCG) and unconjugated estriol (uE₃) in the serum of pregnant women.

The triple screen provides a means to screen the population of pregnant women to determine which pregnancies are at risk for Downs syndrome and other serious genetic defects. The risk is calculated based on the results of the screen, along with other cofactors, such as, maternal age, to determine if the risk is high enough to warrant an invasive diagnostic procedure, such as, amniocentesis, CVS or fetal blood sampling. Such prenatal screens, as the triple screen, can be used either to reduce the need for amniocentesis or to increase Downs syndrome detection for the same number of amniocentesis. "The efficiency of the Triple test is projected to be one case of fetal Downs syndrome detected for every 50 amniocenteses performed." Canick and Knight, "Multiple-marker Screening for Fetal Downs Syndrome," Contemporary OB/GYN, pp. 3-12 (April 1992).

Although pregnant women who are 35 years or older are the standard high risk group for fetal Downs Syndrome, screening also needs to be applied

to young women because although they are at lower risk, most affected pregnancies are in young women. Approximately 80% of babies born with Downs syndrome are born to mothers under 35. ["Downs Syndrome Screening Suggested for Pregnant Women under 35, "ACOG Newsletter, 38(8): 141 (August 1994).]

The triple screen combines the analysis of three serum markers to reduce false positive results (which result in the performance of unnecessary invasive procedures) and false negatives (in which serious genetic defects, such as, trisomy 21, go undetected). In women under 35, the double screen (AFP and hCG) can detect about half of Downs syndrome cases and a large proportion of other chromosome defects during the second trimester. The triple screen (AFP, hCG and uE₃) increases the detection rate of Downs syndrome by 5-10% and a further increase in the detection of all other serious chromosome defects, thus decreasing the number of false-negatives. Such rates mean that the double and triple screens still fail to detect a significant number (30%-35%) of Downs syndrome affected pregnancies.

Other screening markers have been found which may offer some predictive value with respect to Downs Syndrome. The present Applicant has added to this repertoire of predictive markers by finding that leptin, prorenin and/or renin are predictive of a pregnancy being affected by Downs Syndrome.

Leptin has heretofore been associated with obesity. Obesity is the result of a disorder in the body energy balance that occurs when energy intake chronically exceeds energy expenditure. This excess in energy intake is stored in the adipocyte. The recently discovered hormone leptin contributes to the regulation of energy balance by informing the brain of the amount of adipose tissue in the body. The brain may then make the appropriate adjustments in either energy intake or expenditure. Leptin is the protein product of the *ob* gene and in humans is expressed exclusively in adipose tissue. Studies suggest that leptin is a negative regulator of adiposity. However, leptin has only recently been discovered and further investigations into its actions in humans and its role in obesity remain to be determined. Leptin has also heretofore been generally associated with reproductive function.

Renin is an enzyme that belongs to the family of aspartyl proteases, a classification that is based on the properties of having 2 aspartic acid residues at the active site and its susceptibility to inhibition by pepstatin. Renin synthesis was first discovered in the juxtaglomerular cells of the kidney. At present there is evidence that renin synthesis can also occur in other organs such as brain, heart and arterial smooth muscle. Renin circulates in two different forms, prorenin and the active renin form. Prorenin is the enzymatically inactive biosynthetic precursor of renin. In the secretory granules of the juxtaglomerular cell, prorenin is processed to active renin by a thiol protease resembling cathepsin B. An amino terminal prosegment of 42 amino acids is cleaved from the prorenin which allows the exposure of the active site of renin. Active renin converts angiotensinogen (renin substrate) to the biologically inactive decapeptide angiotensin I. Angiotensin I in turn is converted to the octapeptide angiotensin II by means of the angiotensin converting enzyme (ACE). Angiotensin II causes constriction of the small arteries and also promotes sodium and water reabsorption in tubules both directly and indirectly via aldosterone. Aldosterone is a steroid hormone produced by the adrenal gland and its secretion is stimulated by Angiotensin II. Heretofore, the clinical utility of plasma renin is mainly centered around the diagnosis and management of patients with hypertension due to renal artery stenosis or renovascular hypertension. Approximately 10% of the adult population suffers from hypertension. Renal vascular stenosis is the cause of this hypertension in a subgroup of the patients. This subgroup constitutes 1% of the total hypertensive population. A rise in plasma prorenin often precedes the onset of vascular injury in patients with diabetes mellitus. Plasma prorenin measurements may be useful for predicting which patients will develop vascular injury and for monitoring the progression of the disease.

Human chorionic gonadotropin (hCG) stimulation of the ovaries leads to elevated serum prorenin levels. Prorenin levels, like hCG, are high during the first trimester of pregnancy and decrease in the 2nd and 3rd trimesters. Since hCG levels are increased in Downs syndrome pregnancies relative to normal pregnancies and hCG stimulation leads to increased prorenin levels, this led Applicant to postulate that prorenin (or renin) may also be increased in Downs Syndrome pregnancies.

Accordingly, it would be desirable to provide assay methods and compositions for leptin and/or prorenin and/or renin which would have predictive value with respect to the likelihood that a pregnant woman is carrying a fetus having Down's Syndrome.

5

Summary of the Invention

Leptin levels in maternal biological samples are 3-fold higher during pregnancy and correlate positively with human chorionic gonadotropin (hCG) and progesterone levels. hCG levels are increased in Down's syndrome pregnancies relative to normal pregnancies; these facts provided the impetus to the Applicant to determine if the leptin levels correlation with hCG levels may extend to a relative increase in leptin in Down's Syndrome affected pregnancies.

In one aspect, the presently claimed subject matter is directed to a method of determining an increased risk of a woman carrying a Down's Syndrome affected fetus. The method comprising the steps of: quantitatively assaying a sample from a pregnant woman for an amount of leptin in the sample, thereby determining the amount of leptin in the sample; and comparing the amount of leptin in the sample from the pregnant woman with an amount of leptin found in pregnant women carrying a Down's syndrome unaffected fetus and comparing the amount of leptin in the sample from the pregnant woman with an amount of leptin found in pregnant women carrying a Down's syndrome affected fetus, thereby determining those at an increased risk of carrying a Down's syndrome affected fetus.

In a further aspect of the presently claimed subject matter, the amount of leptin in the sample as determined in a sample from a pregnant woman is below the median amount of leptin found in a pregnant women carrying a Down's syndrome unaffected fetus

In one embodiment of the present invention, the sample from the pregnant women is selected from the group consisting of serum, plasma or urine.

In another embodiment of the presently claimed subject matter, the sample is taken from a pregnant women in either of the first trimester or the second trimester or the third trimester of pregnancy.

In a particular embodiment of the presently claimed subject matter, the quantitative assay of the sample is performed by immunoassay, more

particularly a competitive immunoassay or a direct immunoassay. In a particular embodiment, a radioimmunoassay can be used.

5 In another embodiment of the presently claimed subject matter, an additional step of analyzing at least one additional analyte selected from the group consisting of hCG, unconjugated estriol, alpha-fetoprotein, inhibin, PAPP-A, progesterone, DHEA-S or Leukocyte acid phosphatase is performed.

10 In yet another embodiment, an additional step of analyzing at least one additional factor predictive of an increased risk of a fetus being affected by Downs syndrome is performed. In a preferred embodiment, an ultrasound result is the additional predictive factor.

Human chorionic gonadotropin (hCG) stimulation of the ovaries leads to elevated serum prorenin levels. Prorenin levels, like hCG, are high during the first trimester of pregnancy and decrease in the 2nd and 3rd trimesters. Since hCG levels are increased in Downs syndrome pregnancies relative to normal pregnancies and hCG stimulation leads to increased prorenin levels, this led Applicant to postulate that prorenin (or renin) may also be increased in Downs Syndrome pregnancies.

20 In another aspect, the presently claimed subject matter is directed to a method of determining an increased risk of a woman carrying a Downs Syndrome affected fetus. The method comprising the steps of: quantitatively assaying a sample from a pregnant woman for an amount of prorenin in the sample, thereby determining the amount of prorenin in the sample; and comparing the amount of prorenin in the sample from the pregnant woman with an amount of prorenin found in pregnant women carrying a Downs syndrome unaffected fetus and comparing the amount of prorenin in the sample from the pregnant woman with an amount of prorenin found in pregnant women carrying a Downs syndrome affected fetus, thereby determining those at an increased risk of carrying a Downs syndrome affected fetus.

30 In a further aspect of the presently claimed subject matter, the amount of prorenin in the sample as determined in a sample from a pregnant woman is below the median amount of prorenin found in a pregnant women carrying a Downs syndrome unaffected fetus

In one embodiment of the present invention, the sample from the pregnant women is selected from the group consisting of serum, plasma or urine.

In another embodiment of the presently claimed subject matter, the sample is taken from a pregnant women in either of the first trimester or the second trimester or the third trimester of pregnancy.

5 In a particular embodiment of the presently claimed subject matter, the quantitative assay of the sample is performed by immunoassay, more particularly a competitive immunoassay or a direct immunoassay. In a particular embodiment, a radioimmunoassay can be used.

10 In another embodiment of the presently claimed subject matter, an additional step of analyzing at least one additional analyte selected from the group consisting of hCG, unconjugated estriol, alpha-fetoprotein, inhibin, PAPP-A, progesterone, DHEA-S or Leukocyte acid phosphatase is performed.

15 In yet another embodiment, an additional step of analyzing at least one additional factor predictive of an increased risk of a fetus being affected by Downs syndrome is performed. In a preferred embodiment, an ultrasound result is the additional predictive factor.

20 In another aspect, the presently claimed subject matter is directed to a method of determining an increased risk of a woman carrying a Downs Syndrome affected fetus. The method comprising the steps of: quantitatively assaying a sample from a pregnant woman for an amount of renin in the sample, thereby determining the amount of renin in the sample; and comparing the amount of renin in the sample from the pregnant woman with an amount of renin found in pregnant women carrying a Downs syndrome unaffected fetus and comparing the amount of renin in the sample from the pregnant woman with an amount of renin found in pregnant women carrying a Downs syndrome affected fetus, thereby
25 determining those at an increased risk of carrying a Downs syndrome affected fetus.

30 In still further aspects combinations of leptin and/or prorenin and/or renin are assayed and statistical methods of analyzing the contribution of more than two factors to the likelihood of an outcome, as are known in the art, are used to predict those at an increased risk of carrying a Downs syndrome affected fetus.

Other features and advantages of the invention will become apparent from the following detailed description.

Detailed Description of the Invention

A total of 397 second trimester (15-20 weeks gestation) serum samples were collected from individuals with a normal Downs Syndrome risk ($< 1:270$ based on the individual's maternal age, and levels of alpha fetoprotein [AFP], hCG, and unconjugated estriol [uE3]). A total of 10 second trimester serum samples from known Downs Syndrome pregnancies were also collected. All samples were tested blindly for leptin utilizing a radioimmunoassay. It is to be understood that other methods of assay which allow for the quantification of leptin in a biological sample and which are or may become available are within the scope of the present invention.

Leptin values in unaffected pregnancies were both gestational age and maternal age independent. A positive correlation was observed, however, with maternal weight ($R^2 = 0.5345$). Results from the unaffected pregnancies ranged from 1.2 to 93.6 ng/mL (median of 19.5 ng/mL, 1.0 (Multiple of the Median ("MoM")). Results from the 10 Downs Syndrome pregnancies ranged from 2.7 - 44.7 ng/mL (median of 11.7 ng/mL, 0.60 MoM, $p = 0.012$). None of the levels from Downs Syndrome pregnancies exceeded the 95th percentile; however, 2 were below the 5th percentile. Only one exceeded 1.70 multiples of the median (MoM), but levels from 6 of the 10 Downs Syndrome pregnancies were below 0.7 MoM.

The data illustrate that leptin levels are significantly decreased in Downs Syndrome pregnancies relative to unaffected pregnancies. The 0.60 median MoM is lower than that typically reported in the peer reviewed literature for AFP (0.73 MoM) and uE3 (0.72 MoM), but is not quite as large a difference from unaffected pregnancies as is hCG (1.70 MoM). Thus, it appears that leptin is a more sensitive marker for Downs syndrome risk than AFP and uE3, but not quite as sensitive as hCG.

Table 1 - Median of leptin concentration in unaffected cases

Gestational Age (weeks)	n	Median (ng/mL)
15	68	17.4
16	60	19.4
17	71	19.2
18	60	24.1
19	69	17.6

20 68 23.6

All weeks 397 19.5

5 Table 2 – Leptin MoM in Downs syndrome affected and unaffected cases

	Affected Cases	MoM	Affected cases (ng/mL)
	1	0.14	2.7
	2	0.35	6.9
10	3	0.45	8.7
	4	0.51	9.9
	5	0.56	11.0
	6	0.64	12.4
	7	0.74	14.4
15	8	0.77	15.1
	9	1.11	21.6
	10	2.29	44.7
	Mean	0.76	
	Median	0.60	Unaffected - 1.00

20

EXAMPLE 1 - LEPTIN ASSAY

In a particular embodiment of the claimed method, leptin can be quantitated with the *Linco RIA Kit*, available from Linco Research, Inc. 14 Research Park Drive, St. Louis Missouri, 63304. The Linco RIA Kit provides for a competition assay format for assaying for leptin. However, direct immunoassays and other quantitative assay methods as are known in the art or as will be developed are within the scope of the present claims. The standards, Quality Control ("QC") pools, and samples are incubated with the highly specific Rabbit anti-human Leptin in assay buffer and radiolabeled ¹²⁵I-leptin in an equilibrium radioimmunoassay. The assay buffer is, for example, 0.05 M Phosphosaline at pH 7.4, with 0.025M EDTA, 0.1% Sodium azide, 1% RIA grade BSA and 0.05% Triton X-100.

30

The bound/free separation is achieved with the Precipitating Reagent, for example, a Goat anti Rabbit IgG serum in 3%PEG and 0.05% Triton X-100

in 0.05 M Phosphosaline, 0.025 M EDTA, 0.1% sodium azide and centrifugation. The resulting antibody-bound radiolabeled ¹²⁵I-leptin is measure in a gamma counter and the raw data (cpm's) are data reduced, preferably, by a computer program, as would be known to those of ordinary skill in the art, that constructs a standard curve using a dose-response relationship from which the QC pools and samples are read from.

The disclosure herein describes the basic process of data reduction which may be performed manually on raw data. Counted data may also be reduced in part or in whole with assistance of programmable counting equipment or computer processing.

Assay Parameters:

Non-specific binding (NSB)

$$\% \text{ NSB} = \frac{\text{CPM (NSB)}}{\text{CPM (TC)}} \times 100$$

TC = Total Counts

NSB = Non-specific binding

Maximum Binding (Bmax)

$$\% \text{ Bmax} = \frac{\text{CPM (Bo)} - \text{CPM (NSB)}}{\text{CPM (TC)}} \times 100$$

Bmax = maximum binding

Bo = binding in the zero standard
(tubes 5 & 6)

Dose-response variables:

Percent bound for the standards, controls, and specimens, also known as the response.

$$\% \text{ B/Bmax} = \frac{\text{CPM (B)} - \text{CPM (NSB)}}{\text{CPM (Bo)} - \text{CPM (NSB)}} \times 100$$

B = binding for standards, controls, and specimens.

A dose-response curve (DRC) can be constructed using a log-logit transformation of standard dose (concentration) versus % B/Bmax.

Typical Values for adults (18-61 years) who are Lean Subjects with BMI range of 18-25 are as follows:

Adults Males 1.2 - 9.5 ng/mL (n = 59).

Adult Females 4.1 - 25.0ng/mL (n = 60).

BMI = Body Mass Index = $\frac{\text{Body weight in Kilograms}}{(\text{Height in Meter})^2}$ =

5 The control and sample results can be reported to the nearest tenth of a decimal in ng/mL of leptin. The following reference is incorporated herein by reference : Zhongmin, Ma., Gingerich, R.L., et. al. Radioimmunoassay of Leptin in Human Plasma. Clinical Chemistry. 42;6: 942-946, 1996.

10 The most preferred maternal biological sample type is serum, however, plasma is also acceptable. Other biological samples which may be used include, urine, saliva, ascites fluid, peritoneal fluid and other biological fluids.

A total of 418 second trimester (15-20 weeks gestation) serum samples were collected from individuals with a normal Downs Syndrome risk (< 1:270 based on the individual's maternal age, and the levels of alpha fetoprotein [AFP], hCG, and unconjugated estriol [uE₃]). A total of 10 second trimester serum samples from known Downs Syndrome pregnancies were also collected. All samples were tested blindly for prorenin, renin, and total renin in an immunoradiometric assay.

20 Prorenin and renin levels in unaffected pregnancies were gestational age (GA) independent; however, total renin levels were slightly GA dependent (levels decreased with increasing GA). All three analytes were maternal age independent. The following medians set forth in Table 3 were obtained in unaffected and Downs Syndrome pregnancies:

	<u>Unaffected Pregnancies</u>		<u>Affected Pregnancies</u>	
	mU/L	MoM	mU/L	MoM
Prorenin medians	641	1.0	440	0.69
Renin medians	182	1.0	310	1.71
Total renin medians	824	1.0	929	1.15

25 Table 4 - Median Prorenin concentration in unaffected cases

Gestational Age(weeks)	n	Median (mU/L)
15	68	746
16	73	720
17	63	632

	18	75	578
	19	75	647
	20	64	582
5	All weeks	418	641

Table 5 – Prorenin MoM in downs Syndrome affected and unaffected cases

	Affected Cases	MoM	Affected (mU/L)
10	1	0.05	33
	2	0.42	271
	3	0.51	328
	4	0.54	347
	5	0.61	392
15	6	0.76	488
	7	0.82	528
	8	0.96	615
	9	1.17	753
	10	1.30	834
20	Mean	0.71	
	Median	0.69	Unaffected 1.00

Table 6 – Median of Renin concentration in downs syndrome unaffected cases

	Gestational Age (weeks)	n	Median (mU/L)
25	15	67	204
	16	74	187
	17	63	188
	18	74	148
30	19	79	172
	20	63	183
	All weeks	420	180

Table 7 – Renin MoM in Downs syndrome affected and unaffected cases

	Affected Cases	MoM	Affected (mU/L)
	1	0.60	113
5	2	4.35	748
	3	1.44	248
	4	1.31	225
	5	0.68	125
	6	1.94	396
10	7	1.41	265
	8	4.01	819
	9	4.21	858
	10	1.74	355
	Mean	2.41	
15	Unaffected Median 1.00		

Prorenin levels are significantly decreased in Downs Syndrome pregnancies ($p = 0.23$), whereas renin concentrations are increased. The 0.69 and 1.71 median MoMs for prorenin and renin are similar to the MoMs of current prenatal screening markers (0.73 MoM for AFP, 1.70 MoM for hCG, and 0.72 for uE3). Based on this finding, prorenin and renin appear to be useful markers for prenatal Downs Syndrome screening.

The data presented above for leptin and prorenin/renin is given in values of ng/mL and mU/L, respectively. However, by the terms "amount of leptin" or "amount of prorenin" or "amount of renin" is meant any information regarding, for example, either the amount of an analyte present, e.g., in units such as mols, or the weight of an analyte present such as in mg, either of which may be further characterized in relation to their presence per unit volume or weight of a liquid. However, any units and any physical characteristics which allow for the medically or biochemically relevant comparison of different samples with respect to leptin and/or prorenin and/or renin are within the scope of the present invention and the terms "amount of leptin" or "amount of prorenin" or "amount of direct renin".

Additionally, the terms "amount of leptin" or "amount of prorenin" or "amount of direct renin" includes values provided by indirect measurements of

leptin and/or prorenin and/or renin, such as, chemiluminescent, fluorescent, electrical, chemical or otherwise machine or human detectable signals which either provide medically or biochemically relevant information or which can be mathematically manipulated to provide the "amount of leptin" or "amount of prorenin" or "amount of renin" as those terms are defined in the preceding paragraph. Similarly, the use of statistical measurements such as the median can be replaced with other statistical measures as are known in the art. Also, by the term "quantitatively assay" is meant obtaining an actual value for one of leptin or renin or prorenin or use of a means which has a predetermined sensitivity for a given amount of leptin or renin or prorenin. For example, use of a dipstick that gives a human readable signal only when an analyte is above or below a given threshold.

The methods of the present invention can be used in all types of assays, for example, direct, competitive, simultaneous, sequential and sandwich assays as are known in the art are within the scope of the present claims.

EXAMPLE 2 - PRORENIN AND RENIN ASSAY

Renin, Prorenin, and Total Renin are measured by, for example, the Nichols Institute Diagnostics, 33051 Calle Aviador, San Juan Capistrano, CA 92675, BV Active Renin Assay which is a two site radioimmunoassay (IRMA) utilizing two different monoclonal antibodies to human Renin. One monoclonal antibody is coupled to biotin, while the other monoclonal antibody is radiolabeled, with for example ^{125}I , for detection. Renin is "sandwiched" between these two antibodies and this complex is bound to a solid phase avidin coated bead via the high affinity interaction between the biotin and avidin. Lyophilized Standards containing human active renin in sheep serum with 0.1% sodium azide as a preservative can be used. These standards are calibrated by the manufacturer against the World Health Organizations 2nd IRP (68/356) for actual renin. One (1) m U/L obtained using the Active Renin Assay is equivalent to 0.6 pg/mL of WHO 2nd IRP (68/356) for active Renin.

After incubation, the bead is washed to remove unbound components and the radioactivity bound to the solid phase is measured in a gamma counter. The radioactivity of the bound sandwich complex is directly proportional to the amount of immunoreactive renin in the sample. Total Renin is quantitated by adding a Renin "Inhibitor" to the sample which causes the non-

immunoreactive prorenin to become immunoreactive and thus a Total Renin measurement (renin + prorenin) is achieved. Prorenin is therefore calculated by subtracting the Renin measurement from the Total Renin measurement of the sample. The resulting difference is the Prorenin quantitation. Total Renin

5 - Renin = Prorenin

A representative dose curve (RDRC) can be prepared by calculating the mean and ± 2 SD of the cpm for each point of the standard curve in at least 10 acceptable assays.

10 The disclosure herein describes the basic process of data reduction which may be performed manually on raw data. Counted data may also be reduced in part or in whole with assistance of programmable counting equipment or computer processing.

15 The CPM of each standard dose replicate is plotted against the standard dose concentration (Linear vs. Linear). A computer program draws a smooth point to point curve using a Spline Curve Fitting reduction. Sample doses are read off the Spline Dose Response Curve (DRC) for each CPM replicate. The computer then averages the read doses (duplicate) and calculates the mean dose and %CV. Control and Sample values are averaged to the nearest whole number. Prorenin measurements are calculated by subtracting the Renin from the Total Renin measurements.

Normal Values for adults are as follows:

25	Renin:	12 - 79 mU/L	Supine Adult
		13 - 114 mU/L	Upright Adult
	Prorenin:	57 - 285 mU/L	21-35 Years
		48 - 224 mU/L	> 36 Years

Total Renin:	64 - 325mU/L	Adults
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30 The following references are incorporated herein by reference: Hsueh WA and Baxter JD. Human prorenin. Hypertension 1991; 17:469; Derkx FHM, Stuenkel C, Schalekamp MPA, Visser W, Huisveld IH and Schalekamp MADH. Immunoreactive renin, prorenin and enzymatically active renin in plasma during pregnancy and in women taking oral contraceptives. J. Clin.

Endocrinol. Metab. 1986; 63:1008; Sealey JE. Plasma Renin Activity and Plasma Prorenin Assays. Clin. Chem. 1991;37/10(B), 1811-1819; Heusser CH, Bews JPA, Alkan SS, Dietrich FM, Wood JM, de Gasparo M, and Hofbauer KG. Monoclonal antibodies to human renin: properties and applications. Clin. Exper. Theory Practice 1987; A9(8&9):1259-1275; Zuo WM, Pratt RE, Heusser CH, Bews JPA, de Gasparo M, and Dzau VJ. Characterization of a monoclonal antibody specific for human active renin. Hypertension 1992; 19:249-254; Simon D, Hartmann DJ, Badouaille G, Caillot G, Guyenne TT, Corvol P, Pau B, Marchand J. Two-Site direct Immunoassay Specific for Active Renin. Clin. Chem. 1992;38/10, 1959-1962; Rodbard, D., and Hutt, D.: Statistical Analysis of Radioimmunoassays and Immunoradiometric (labeled antibody) Assay. Radioimmunoassays and Related Procedures in Medicine. Vol. 1, Vienna: International Atomic Energy Agency, Vienna, 1974.

The most preferred biological sample type is serum, however, plasma is also acceptable. Other biological samples which may be used include, urine, saliva, ascites fluid, peritoneal fluid and other biological fluids.

The presently disclosed embodiments are to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims, rather than the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

What is claimed is:

1. A method of determining an increased risk of a woman carrying a Downs Syndrome affected fetus, the method comprising the steps of:
 - 5 a) quantitatively assaying a sample from a pregnant woman for an amount of leptin in the sample, thereby determining the amount of leptin in the sample; and
 - b) comparing the amount of leptin in the sample in step a with an amount of leptin found in pregnant women carrying a Downs syndrome unaffected fetus and comparing the amount of leptin in the sample in step a with an amount of leptin found in pregnant women carrying a Downs syndrome affected fetus, thereby determining those at an increased risk of carrying a Downs syndrome affected fetus.
- 15 2. The method of claim 1 wherein the amount of leptin in the sample as determined in step a is below the median amount of leptin found in a pregnant women carrying a Downs syndrome unaffected fetus
- 20 3. The method of claim 1 wherein the sample from the pregnant women is selected from the group consisting of serum, plasma or urine.
4. The method of claim 1 wherein the sample is taken from a pregnant women in either of the first or the second or the third trimester of pregnancy.
- 25 5. The method of claim 1 wherein the quantitative assay of the sample is performed by immunoassay.
6. The method of claim 1 further comprising the step of analyzing at least one additional analyte selected from the group consisting of hCG, unconjugated estriol, alpha-fetoprotein, inhibin, PAPP-A, progesterone, DHEA-S or Leukocyte acid phosphatase.
- 30 7. The method of claim 1 further comprising the step of analyzing at least one additional analyte predictive of an increased risk of a fetus being affected by Downs syndrome.
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8. The method of claim 1 further comprising the step of analyzing at least one additional factor predictive of an increased risk of a fetus being affected by Downs syndrome.
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9. The method of claim 8 wherein the additional factor is an ultrasound result.
10. A method of determining an increased risk of a woman carrying a Downs Syndrome affected fetus, the method comprising the steps of:
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- a) quantitatively assaying a sample from a pregnant woman for an amount of prorenin in the sample, thereby determining the amount of prorenin in the sample; and
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- b) comparing the amount of prorenin in the sample in step a with an amount of prorenin found in pregnant women carrying a Downs syndrome unaffected fetus and comparing the amount of prorenin in the sample in step a with an amount of prorenin found in pregnant women carrying a Downs syndrome affected fetus, thereby determining those at an increased risk of carrying a
- 20
- Downs syndrome affected fetus.
11. The method of claim 10 wherein the amount of prorenin in the sample as determined in step a is below the median amount of prorenin found in a pregnant women carrying a Downs syndrome unaffected fetus.
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12. The method of claim 10 wherein the sample from the pregnant women is selected from the group consisting of serum, plasma or urine.
13. The method of claim 10 wherein the sample is taken from a pregnant women in either of the first trimester or the second trimester or the third trimester of pregnancy.
- 30
14. The method of claim 10 wherein the quantitative assay of the sample is performed by immunoassay.

15. The method of claim 10 further comprising the step of analyzing at least one additional analyte predictive of an increased risk of a fetus being affected by Downs syndrome.
- 5 16. A method of determining an increased risk of a woman carrying a Downs Syndrome affected fetus, the method comprising the steps of:
- a) quantitatively assaying a sample from a pregnant woman for an amount of renin in the sample, thereby determining the amount of renin in the sample; and
- 10 b) comparing the amount of renin in the sample in step a with an amount of renin found in pregnant women carrying a Downs syndrome unaffected fetus and comparing the amount of renin in the sample in step a with an amount of renin found in pregnant women carrying a Downs syndrome affected fetus, thereby
- 15 determining those at an increased risk of carrying a Downs syndrome affected fetus.
17. The method of claim 16 wherein the amount of renin in the sample as determined in step a is below the median amount of renin found in a pregnant
- 20 women carrying a Downs syndrome unaffected fetus.
18. The method of claim 16 wherein the sample from the pregnant women is selected from the group consisting of serum, plasma or urine.
- 25 19. The method of claim 16 wherein the sample is taken from a pregnant women in either of the first trimester or the second trimester or the third trimester of pregnancy.
20. The method of claim 16 wherein the quantitative assay of the sample
- 30 is performed by immunoassay.
21. A method of determining whether there is an increased risk of a woman carrying a fetus which is affected by Downs Syndrome, the method comprising the steps of:

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- a) quantitatively assaying a sample from a pregnant woman for an amount of leptin in the sample, thereby determining the amount of leptin in the sample;
 - b) comparing the amount of leptin in the sample in step a with an amount of leptin found in pregnant women carrying a Down's syndrome unaffected fetus and comparing the amount of leptin in the sample in step a with an amount of leptin found in pregnant women carrying a Down's syndrome affected fetus,
 - c) quantitatively assaying a sample from a pregnant woman for an amount of prorenin in the sample, thereby determining the amount of prorenin in the sample; and
 - d) comparing the amount of prorenin in the sample in step a with an amount of prorenin found in pregnant women carrying a Down's syndrome unaffected fetus and comparing the amount of prorenin in the sample in step a with an amount of prorenin found in pregnant women carrying a Down's syndrome affected fetus, thereby determining those at an increased risk of carrying a Down's syndrome affected fetus.
22. The method of claim 21 wherein the sample from the pregnant women is selected from the group consisting of serum, plasma or urine.
23. The method of claim 21 wherein the sample is taken from a pregnant women in either of the first trimester or the second trimester or the third trimester of pregnancy.
24. The method of claim 21 wherein the quantitative assay of the sample is performed by immunoassay.
25. The method of claim 21 further comprising the step of analyzing at least one additional analyte predictive of an increased risk of a fetus being affected by Down's syndrome.

26. A method of determining whether there is an increased risk of a woman carrying a fetus which is affected by Downs Syndrome, the method comprising the steps of:

- b) quantitatively assaying a sample from a pregnant woman for an amount of renin in the sample, thereby determining the amount of renin in the sample;
- b) comparing the amount of renin in the sample in step a with an amount of renin found in pregnant women carrying a Downs syndrome unaffected fetus and comparing the amount of renin in the sample in step a with an amount of renin found in pregnant women carrying a Downs syndrome affected fetus,
- c) quantitatively assaying a sample from a pregnant woman for an amount of prorenin in the sample, thereby determining the amount of prorenin in the sample; and
- d) comparing the amount of prorenin in the sample in step a with an amount of prorenin found in pregnant women carrying a Downs syndrome unaffected fetus and comparing the amount of prorenin in the sample in step a with an amount of prorenin found in pregnant women carrying a Downs syndrome affected fetus, thereby determining those at an increased risk of carrying a Downs syndrome affected fetus.

27. The method of claim 26 wherein the sample from the pregnant women is selected from the group consisting of serum, plasma or urine.

28. The method of claim 26 wherein the sample is taken from a pregnant women in either of the first trimester or the second trimester or the third trimester of pregnancy.

29. The method of claim 26 wherein the quantitative assay of the sample is performed by immunoassay.

30. The method of claim 26 further comprising the step of analyzing at least one additional analyte predictive of an increased risk of a fetus being affected by Downs syndrome.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/00653

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : G01N 33/48, 33/00, 33/53, 33/573; C12Q 1/00
US CL : 36/ 86, 814, 63, 87, 817, 818; 435/ 7.1, 7.4, 4

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/ 86, 814, 63, 87, 817, 818; 435/ 7.1, 7.4, 4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, DERWENT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Saule et al. Psuedohypoaldosteronism in a Child With Down Syndrome: Long-term Management of Salt Loss by Ion Exchange Resin Administration. Eur J Pediatr. September 1984. Vol. 142(4). pages 286-9.	1-30

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

24 APRIL 2000

Date of mailing of the international search report

13 JUN 2000

Name and mailing address of the ISA/US
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/00653

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/00653

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-9 and 21-25, drawn to a method of determining an increased risk of a woman carrying a Down's Syndrome affected fetus, the method comprising the steps of quantitatively assaying a sample from a pregnant woman for an amount of leptin.

Group II, claim(s) 10-20 and 26-30, drawn to a method of determining an increased risk of a woman carrying a Down's Syndrome affected fetus, the method comprising the steps of quantitatively assaying a sample from a pregnant woman for an amount of prorenin.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Groups I and II do not have the same special technical feature, which is in the instant application, the assaying for leptin.